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DELIVERY OF BIOLOGICS TO THE RETINAL PIGMENT EPITHELIUM

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ACADEMIC DISSERTATION

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ABSTRACT

Biologics are increasingly used in the treatment of ocular diseases such as age-related macular degeneration (AMD) that cannot be controlled with conventional small molecule drugs. AMD is a multifactorial eye disease that carries significant risk of morbidity and vision loss. In Finland and other western countries, AMD affects one in three people older than 75 years, and until the early 2000s no effective treatment was available for these patients. The marketing approval of anti-VEGF antibodies was a major breakthrough in the management of AMD; indeed these biologics effectively halt choroidal neovascularization and therefore prevent further vision loss in roughly half of the patients with wet AMD. Antibody therapy has been the most successful approach so far, however, other biological therapies such as gene therapy, cell therapy and other therapeutic proteins, may prove beneficial in the treatment of AMD and other vision threatening disorders. This thesis deals with the delivery of biologics, including DNA, cells, proteins and peptides, to the retinal pigment epithelium (RPE), which plays a central role in the development of AMD. Briefly, the main topics and results of this work are presented.

New non-viral gene delivery candidates are usually screened for transfection efficiency and toxicity by reading out transgene expression levels relative to a reference formulation after *in vitro* transfection. The screening protocols, however, can be very different among laboratories, so that comparison of results is often difficult, if not impossible (van Gaal et al., 2011). Our aim was to develop a standardized protocol optimized for the transfection of retinal pigment epithelial cells *in vitro*. The developed screening protocol provides a relatively simple and reproducible procedure for the pre-selection of potential candidate reagents as non-viral gene delivery systems targeted to the retinal pigment epithelium.

The ocular delivery of biologics remains a challenging task due to the barriers of the eye. Short cationic peptides, also known as cell-penetrating peptides (CPPs), have been successfully used as tools to introduce various biologics into cells due to their ability to translocate across the plasma membrane and deliver their cargoes intracellularly. In our work, we have explored the functionality of Tat peptide, one of the most widely studied CPPs. Our results indicate that it is not the sequence of Tat *per se* that dictates cell uptake, but the cationic charge of the peptide. Moreover no direct penetration was observed; instead all the peptides were endocytosed and, as it is often the case in non-viral gene delivery, ended their journey inside lysosomes. For this reason, we think that the use of Tat peptide for the delivery of biologics to the cytoplasm or nucleus of cells will probably not be very successful.

Ocular stem cell therapy holds promise for the reconstruction of the degenerated RPE monolayer in AMD patients; in addition, engineered human RPE constructs may also provide a unique platform for drug discovery and toxicology. We have grown a functional RPE tissue *in vitro* by using human embryonic stem cells as cell source and the synthetic polymer polyimide as supporting scaffold for the growth and maturation of the cells. The epithelia acquired RPE-like properties, including characteristic RPE phenotype, expression of RPE markers, barrier and phagocytic function.

The degeneration of RPE cells in dry AMD is caused by the aggregation of proteins inside RPE cells, and is currently untreatable. We have investigated the cytoprotective properties of heat shock protein 70 kDa (Hsp70) against oxidative damage and the feasibility of rhHsp70 protein therapy as a potential therapeutic approach for dry AMD. This work provides a novel therapeutic option for the treatment of RPE degeneration in AMD.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on four publications:

I Subrizi A, Yliperttula M, Tibaldi L, Schacht E, Dubruel P, Joliot A, and Urtti A. Optimized transfection protocol for efficient *in vitro* non-viral polymeric gene delivery to human retinal pigment epithelial cells (ARPE-19). *Protocol Exchange* 2009, Nro 78.
DOI: 10.1038/nprot.2009.78

II Subrizi A, Tuominen E, Bunker A, Róg T, Antopolsky M, and Urtti A. Tat(48-60) peptide amino acid sequence is not unique in its cell penetrating properties and cell-surface glycosaminoglycans inhibit its cellular uptake. *Journal of Controlled Release* 2012, 158(2), 277-285.
DOI: 10.1016/j.jconrel.2011.11.007

III Subrizi A, Hiidenmaa H, Ilmarinen T, Nymark S, Dubruel P, Uusitalo H, Yliperttula M, Urtti A, and Skottman H. Generation of hESC-derived retinal pigment epithelium on biopolymer coated polyimide membranes. *Biomaterials* 2012, 33(32), 8047-8054.
DOI: 10.1016/j.biomaterials.2012.07.033

IV Subrizi A, Toropainen E, Ramsay E, Airaksinen AJ, Kaarniranta K, and Urtti A. Oxidative stress protection by exogenous delivery of rhHsp70 chaperone to the retinal pigment epithelium (RPE), a possible therapeutic strategy against RPE degeneration. *Pharmaceutical Research* 2014.
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ABBREVIATIONS

AAV	Adeno-associated virus
AMD	Age-related macular degeneration
AREDS	Age-related eye disease study
ARPE-19	Spontaneously arising retinal pigment epithelia (RPE) cell line
BEST	Bestrophin-1
CNTF	Ciliary neurotrophic factor
CNV	Choroidal neovascularization
CPP	Cell penetrating peptide
CRALBP	Cellular retinaldehyde-binding protein
DNA	Deoxyribonucleic acid
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
EBNA-1	Epstein–Barr virus nuclear antigen 1
EBV	Epstein–Barr virus
ECT	Encapsulated cell technology
EF1a	Human elongation factor-1 alpha
EMA	European medicines agency
Fc receptor	Receptor with binding specificity to the *fragment crystallisable* region of an antibody
FDA	Food and drug administration
FGF	Fibroblast growth factor
GALA	Synthetic pH-responsive amphipathic peptide
GDNF	Glial cell-derived neurotrophic factor
hES(C)	Human embryonic stem cells
HPMA	N-(2-hydroxypropyl)methacrylamide
Hsp70	Heat shock protein 70 kDa
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin G
LDL	Low-density lipoprotein
LEDGF	Lens epithelium-derived growth factor
MERTK	C-mer proto-oncogene tyrosine kinase
MITF	Microphthalmia-associated transcription factor
OriP	Epstein-Barr Virus replication origin
PBuA	Poly(butyl acrylate)
PDGF	Platelet-derived growth factor
PDMAEMA	Poly 2-(dimethylamino)ethyl methacrylate
PEDF	Pigment epithelium-derived factor
PEG	Polyethylene glycol
PEI	Polyethylenimine
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)

PLGA	Poly(lactic-*co*-glycolic acid)
PMEL	Pre-melanosome protein 17
PS	Protamine sulphate
RCS rat	Royal college of surgeons rat
RGD	Arginylglycylaspartic acid tripeptide
RNA	Ribonucleic acid
RPE	Retinal pigment epithelium
RPE65	Retinal pigment epithelium-specific 65 kDa protein
S/MAR	Scaffold/Matrix attachment region
SV40	Simian virus 40
TALEN	Transcription activator-like effector nuclease
Tat	Trans-activator of transcription
TGF-β	Transforming growth factor beta
TYR	Tyrosinase
VEGF	Vascular endothelial growth factor
ZO-1	Tight junction protein 1

1 INTRODUCTION

Biologics are pharmaceutical products manufactured from living organisms, such as a microorganism, or plant or animal cells, by genetic engineering. They are characterized by their very large size, which is often 200 to 1000 times the size of a small molecule drug, and by their high complexity. Moreover due to their size and sensitivity, biologics are almost always administered parenterally rather than orally like most small molecule drugs. The term biologics is often equated with therapeutic proteins synthesised in engineered systems. More recently, however, nucleic acids used in gene therapy and antisense technology, and cells employed in cell therapy with the aim to restore, maintain, or improve tissue function, gained in importance and they too are generally included in the definition. The first biologic to gain marketing approval was humulin, a recombinant human insulin developed and marketed by Genentech and Eli Lilly, initially approved in the United States in 1982 (Johnson, 1983). In the last 15 years, the pharmaceutical industry has undergone a "biologics boom"; by 2010 some 200 biologics had gained marketing approval (Walsh, 2010), commanding an estimated global market of about $ 115 billion (including vaccines). Millions of patients worldwide benefit from biologic drugs, which are employed in the treatment of a variety of cancers, infectious diseases, inflammatory diseases, autoimmune disorders, cardiovascular diseases, blood disorders, and diabetes.

Age-related macular degeneration (AMD) is a vision threatening disorder of the posterior eye that affects millions of elderly patients worldwide. Although the disease rarely results in complete blindness and peripheral vision may remain unaltered, central vision is gradually blurred, severely affecting ordinary daily activities (de Jong, 2006). AMD is a disease with limited treatability; however, with the advent of antiangiogenic therapy with biologics, in about half of the patients suffering from neovascular AMD, the progression of the disease can be halted (Rosenfeld et al., 2006). Early detection is the key to a successful therapy, because anti-VEGF treatment may be able to prevent the growth of new blood vessels, but it cannot restore vision in an eye with scarring. Nevertheless with the therapy advances of the past 10 years, millions of patients in the early stage of wet AMD, whose retinal architecture is not yet compromised, were able to preserve ocular health, quality of vision, and independence. In order to achieve retinal targeting and an effective therapeutic concentration, biologics need to be administered by direct intravitreal injection. Monthly intravitreal injections of anti-VEGF antibodies/antibody fragments are the standard care for wet AMD patients, however this administration route, in addition to being uncomfortable for the patient, may also cause several adverse effects, including development of endophthalmitis, rise in intraocular pressure,

cataract formation, and increased risk of retinal detachment (Falavarjani and Nguyen, 2013). Hence there is an unmet medical need for new technologies that can provide controlled, scalable and sustained release of biologics, through non-invasive or minimally invasive routes to the back of the eye. This thesis deals with the delivery of biologics, including DNA, cells, proteins and peptides, to the retinal pigment epithelium, which plays a central role in the development of AMD.

2 REVIEW OF THE LITERATURE

2.1 THE RETINAL PIGMENT EPITHELIUM IN HEALTH AND DISEASE

2.1.1 THE RETINAL PIGMENT EPITHELIUM

Vision is the ability to detect, recognize and discriminate objects in space. Our visual system is a highly complex arrangement for analysing information coming from a wide array of signals, all of which are captured by the retinal sensory receptors (Forrester et al., 2008): the photoreceptors. The photoreceptors detect light signals and convert them first to biochemical signals and then to electric stimuli, which are transmitted via several other neurons of the retina to the brain. The photoreceptors are the light sensitive cells in the retina, and yet even the simplest light detecting organs are composed also of a second cell type, the pigmented cell. Both cell types appear together in every eye of the animal kingdom from insects to higher vertebrates (Lamb et al., 2007, Kolb et al., 2014), and their interaction is essential for visual function. The retinal pigment epithelium (RPE) is derived from the same neural tissue that forms the neurosensory retina, although while the retina differentiates into several layers of neurons, the RPE remains a monolayer with characteristics of a secretory epithelium (table 1) (Marmor and Wolfensberger, 1998). The RPE is located in the back of the eye, its apical side facing the photoreceptors of the neural retina and its basolateral surface resting on the Bruch's membrane, a pentalaminar, 1-4 μm thick structure overlaying the fenestrated choroidal capillaries of the eye (figure 1).

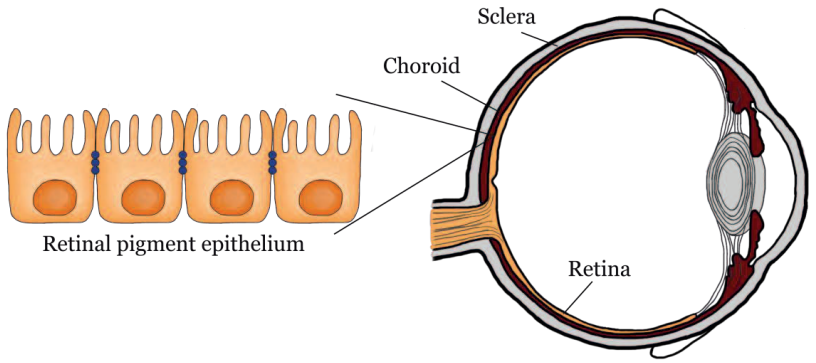

Figure 1 **Sagittal section of the human eye and detail of the retinal pigment epithelium.** The RPE is sandwiched between the retina and the choroid in the posterior part of the eye.

The RPE is a cuboidal (hexagonal from above), post-mitotic, single sheet of pigmented cells joined apically by tight junctions which block the free passage of water and ions. This junctional barrier forms the outer blood-retinal barrier that plays a crucial role in maintaining the viability and function of the neural retina. The RPE exerts several essential supportive functions of homeostasis in the neural retina (see table 1) hence, it is not surprising that RPE impairment plays a central role in the pathogenesis of several degenerative retinal disorders that lead to irreversible vision loss.

Table 1. *Physiologic functions of the RPE*

a)	**Light absorption**
	Melanosomes absorb scattered light and improve the quality of the optical system
	Protection from photo-oxidative damage (antioxidants)
b)	**Blood-retinal barrier**
	Epithelial transport of nutrients and ions
	Water and metabolic end products removal from the subretinal space
	Immune privilege
c)	**Visual cycle**
	Isomerization of all-trans retinal to 11-cis retinal
	Storage of 11-cis retinal
d)	**Secretion of growth factors**
	Essential for maintenance of the retina and choriocapillaris, e.g. fibroblast growth factors (FGFs), transforming growth factor-β (TGF-β), insulin-like growth factor-I (IGF-I), ciliary neurotrophic factor (CNTF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), lens epithelium-derived growth factor (LEDGF), members of the interleukin family, and pigment epithelium-derived factor (PEDF) (Strauss, 2005)
e)	**Phagocytosis of photoreceptor outer segments**
	Diurnally regulated process that is essential for the renewal of photo-oxidized photoreceptor outer segment tips

2.1.2 AGE-RELATED MACULAR DEGENERATION

The RPE is involved in a variety of congenital, inherited, and metabolic disorders, although few have been defined in terms of specific cellular dysfunction at the level of the RPE (Marmor and Wolfensberger, 1998). Degeneration of RPE cells and disruption of the RPE-photoreceptor interface are the cause of loss of macular function in age-related macular degeneration (AMD), the leading cause of blindness in the elderly worldwide. AMD accounts for 8.7% of global blindness and its prevalence is likely to increase as a consequence of exponential population ageing. The projected number of people with the disease is 196 million in 2020, increasing to 288 million in 2040 (Wong et al., 2014).

AMD is characterized by a progressive loss of central vision attributable to degenerative and neovascular changes in the macula, the highly specialized region of the retina responsible for fine visual acuity. The macula constitutes only a small part of the retinal area (about 4%), but it accounts for almost 10% of the entire visual field (Kolb et al., 2014). The typical signs of early AMD are pigmentary changes and/or the appearance of white-yellow deposits called drusen in the fundus of patients. Drusen, whose origin is still unknown, are aggregates of lipids, proteins and extracellular material that accumulate between the RPE and the Bruch's membrane (Crabb et al., 2002). Late AMD is characterized by diffuse atrophy of RPE cells (dry AMD) and/or choroidal neovascularization (wet AMD); if left untreated, wet AMD usually causes legal blindness within months after the second eye becomes affected, in contrast, these events may take years in patients with dry AMD. AMD is a complex multifactorial disease and the reasons for RPE dysfunction have not yet been identified, although a variety of possible causes have been recognized including genetic factors (genetic contribution is identified in up to 25% of AMD cases) (Seddon et al., 1997), ischemia, oxidative stress, phagocytic overload, cigarette smoke, lipofuscin toxicity, inflammation, and microbial infection (Zarbin, 2004).

Therapeutic approaches to AMD have been mostly focused on the wet form of the disease, because the pathogenic mechanisms of dry AMD are still unclear. Wet AMD is currently treated with the intraocular administration of anti-neovascular agents that block VEGF binding to its receptor on endothelial cells, thus preventing neovascularization. Moreover the risk of progression to advanced neovascular AMD is reduced with the oral supplementation of high levels of antioxidants and zinc (AREDS formulation: vitamin C, vitamin E, β-carotene, and zinc) (Kassoff et al., 2001). While intravitreal antiangiogenic therapy has been the most effective therapeutic strategy so far, it is beneficial only for less than 50% of patients with wet AMD, and therefore less than 5% of all AMD patients (Rosenfeld et al., 2006, Kolb et al., 2014, Gragoudas et al., 2004, Algvere et al., 2008). Thus, despite the significant advances with anti-VEGF therapy in slowing the progression of wet AMD, there is still a large unmet medical need for many patients who have already lost vision from this condition and for the vast majority of AMD patients suffering from RPE geographic atrophy.

2.2 WHAT ARE BIOLOGICS?

Biologics, also called biopharmaceuticals, are medicinal products produced by biotechnology comprised of proteins such as hormones, enzymes, or monoclonal antibodies, but also gene and cell therapy products. Like all medicines, biologics work by interacting with the body to produce a therapeutic outcome. Many, but not all biologics are made using genetically modified cells; their complex manufacturing processes are very sensitive, and

precise control is required to obtain consistent results and to guarantee the safety and efficacy of the final product. Recombinant human insulin was the first biologic approved in the United States in 1982. Prior to that, protein products approved for use in humans were extracted from natural sources (Samanen, 2013). Biologics are the fastest growing sector of the pharmaceutical industry, with total global market sales in 2011 of about $ 115 billion (including vaccines), which represents about 16% of the gross 2011 prescription drug sales estimated at about $ 710 billion (Carton and Strohl, 2013).

Having defined what biologics are, the second question that arises is how do they differ from synthetic drugs? The key differences lie in the size, manufacturing techniques, physicochemical properties, pharmacokinetics and pharmacodynamics properties, and route of administration (table 2).

Table 2. *Key differences between biologics and synthetic drugs.*

Property	Biologics	Synthetic drugs
Size	Large, > 1000 Da	Small, < 500 Da
Manufacture	Biologically produced	Chemical synthesis
Physicochemical properties	Complex and variable, undergo post-transcriptional modifications, e.g. glycosylation	Mostly well defined
Route of administration	Parenteral administration	Oral administration

Genentech has compared the difference in size and complexity between aspirin (21 atoms) and an antibody (>20 000 atoms) to the difference between a bike (10 kg) and a business jet (> 10 000 kg). This example well portrays the problems encountered in the delivery of biologics to the site of action; their large size prevents them from crossing biological barriers, and their inherent physicochemical complexity makes them unstable and prone to degradation. Consequently, there is a compelling need to design delivery systems that allow the safe and effective delivery of biologics to their site of action. In the following chapters the ocular delivery of selected biologics, including genes, cells, and recombinant therapeutic proteins, will be discussed in more detail.

2.3 GENE THERAPY

2.3.1 THE ROCKY ROAD TO GENE THERAPY

Gene therapy is a medical procedure that attempts to correct a genetic defect (such as a lacking or malfunctioning gene sequence), to increase the production of a therapeutic protein or to render cells susceptible to the

body's own defence mechanisms or drug treatment, by replacing a defective gene with a normal gene. The major benefit of gene therapy is that it has the potential to provide a cure after a single intervention, since its beneficial effects may last a lifetime. The idea of treating human disease at a genetic level began to form in the mid-1960s, when the work with tumorigenic viruses of Renato Dulbecco and colleagues (Sambrook et al., 1968) established that in the course of transforming a cell from the normal to the neoplastic phenotype, the papovaviruses SV40 and polyoma integrated their genetic information stably and heritably into the genomes of target cells (Friedmann, 1992). This observation together with advances in DNA manipulation techniques, led to proposal that exogenous "good" DNA may be used to replace the defective DNA in those who suffer from genetic defects (Friedman and Roblin, 1972).

The first gene therapy clinical trial was approved in 1989 (Rosenberg et al., 1990) and in the following years several other trials followed, until in 1999 an 18-year-old trial participant who had an unusually mild form of liver disease caused by mutations in a gene on the X chromosome, died 4 days after receiving an injection of an adenovirus carrying the corrected gene (Raper et al., 2003). This death and other tragic adverse events, including treatment-induced leukaemia in some volunteers (Hacein-Bey-Abina et al., 2008), suddenly debunked the initial excitement generated by the advent of gene therapy. Fortunately, despite many difficulties and waning interest in the field, gene therapy has recently experienced a revival among scientists and pharmaceutical companies, due to successful clinical trials for several types of immunodeficiency diseases (Gaspar et al., 2011a, Gaspar et al., 2011b), haemophilia (Nathwani et al., 2011), cancer (Kaufman et al., 2010, Porter et al., 2011, Kalos et al., 2011), and eye disorders (Bainbridge et al., 2008, Maguire et al., 2008, Cideciyan et al., 2008, MacLaren et al., 2014). Moreover in 2012 the European Medicines Agency approved the marketing application of Glybera, an adeno-associated virus engineered to express lipoprotein lipase in the muscle for the treatment of lipoprotein lipase deficiency, and first gene therapy treatment to win approval by western regulators.

2.3.2 AN EYE ON GENE MEDICINES

Ophthalmology is one of the branches of medicine where gene medicines show great promise and, in recent years gene therapy has emerged as a novel approach for the treatment of many retinal disorders that are considered incurable. The eye offers several advantages as a target for gene therapy, since a) it is an immune-privileged site, thus the likelihood of systemic immune response is decreased, b) it is small and well defined, this allows for localized treatment with smaller doses, and consequently reduced chance of systemic absorption and toxicity, and c) the effects of localized ocular treatments can be easily observed and monitored for efficacy.

The only gene medicine approved so far for ocular treatment is pegaptanib (Macugen), a 28-base RNA aptamer, developed to bind and block the activity of extracellular VEGF, specifically the 165-amino-acid isoform (VEGF165) (Gragoudas et al., 2004). In order to allow for a 6 weeks administration interval, the aptamer is covalently linked to two branched 20 kDa PEG moieties that increase its intravitreal half-life and the sugar backbone has been modified to prevent nuclease degradation (Ruckman et al., 1998). To date, 31 gene therapy clinical trials for ocular diseases, including AMD, choroideremia, Leber's hereditary optic neuropathy, retinitis pigmentosa, Leber's congenital amaurosis, superficial corneal opacity, glaucoma, Stargardt's disease, and diabetic macular edema, have been initiated (source The Journal of Gene Medicine Clinical Trial site). While most trials are still in the early phases, one AAV-based gene therapy trial for Leber's congenital amaurosis is currently in advanced development with safety and efficacy being evaluated in a phase III clinical trial.

Viral gene therapy is still the most popular choice for gene therapies now in development, and adeno-associated virus (AAV) is the most successful vector used so far. Indeed, viruses such as lentiviruses, adenoviruses, retroviruses and the aforementioned adeno-associated viruses, work as natural syringes that inject genetic material into cells very effectively. However, despite being stripped of disease-causing elements, viruses are pathogens and safety concerns, together with difficulties of production on a large scale and low packaging capacity, have led to the search of alternative approaches.

Non-viral gene delivery systems have the potential to provide nucleic acid-based therapeutics that closely resemble traditional pharmaceuticals. That is, the products should be 1) capable of being administered repeatedly with little immune response, 2) produced in large quantities with high reproducibility and acceptable cost, 3) stable to storage, and 4) easy to administer to patients (Davis, 2002). Other advantages of non-viral methods include very low frequency of integration, and the possibility to pack very large genes into the delivery system. Notwithstanding these favourable conditions, the widespread use of non-viral gene delivery systems is still hampered by a lack of efficacy, especially *in vivo*, attributable to the inability of these vectors to overcome the numerous barriers encountered between the site of administration and intracellular localization.

2.3.3 MAKING NON-VIRAL SYSTEMS MORE EFFECTIVE

Gene delivery *in vitro* is hampered by barriers present both outside and inside cells. Shortcomings arise due to the physicochemical characteristics of the genetic material itself, including susceptibility to degradation by nucleases, presence of bacterial motifs (i.e. in plasmid DNA), large size, and highly negative charge. Intracellular barriers encountered by non-viral gene delivery systems include low cellular uptake, entrapment inside lysosomes,

cytoplasmic degradation and poor translocation kinetics, low nuclear targeting (for gene therapeutics requiring nuclear entry), and loss of genetic material leading to short gene expression. Moreover, the majority of gene delivery systems are cationic and therefore prone to non-specific interactions with negatively charged cell surface molecules such as glycosaminoglycans.

These non-specific interactions will have deleterious effects *in vivo* and lead to biased biodistribution, compromised stability, and lack of cellular specificity. Strategies to enhance the efficacy, specificity, and temporal control of non-viral gene delivery rely on the improvement of the delivery vectors as well as the modification of DNA itself (summarized in table 3).

Table 3. *Genetic material optimization and vector design can improve transgene expression; commonly used strategies to overcome transfection bottlenecks.*

Optimization of genetic material		**Vector design**	
Problem	Solution	Problem	Solution
Loss of plasmid	Episomal replication (i.e. EBNA-1, S/MAR)	**Poor uptake**	Decrease size
	Site specific integration (i.e. TALENs, transposons)		Membrane penetrating agents (i.e. CPPs)
Promoter shutdown	Alternative promoters (i.e. non-viral promoters, cell specific promoters)		Receptor-mediated uptake (i.e. CD44, transferrin, LDL)
Bacterial motifs[1]	Minimize GC content	**Lysosomal entrapment**	Pore forming peptides (i.e. toxins, CPPs)
	Remove bacterial backbone (minicircle DNA)		Fusogenic lipids and peptides (i.e. DOPE, GALA)
DNA degradation	DNA topology (supercoiled DNA)		pH-buffering effect (i.e. PEI)
	Nucleic acid analogs (i.e. morpholino)	**Low nuclear import**	Nuclear localization signal (i.e. Tat)
Low nuclear import	Nuclear localization signal (i.e. SV40 enhancer)	**Non-specific interactions**	Shielding moieties (i.e. PEG, HPMA)
			Active targeting (i.e. RGD)

[1] Can elicit inflammation, cell death, and silencing (Mitsui et al., 2009)

2.4 CELL THERAPY AND TISSUE ENGINEERING

2.4.1 GROWING NEW TISSUES TO REPLACE MALFUNCTIONING ONES

In cell therapy, cells are injected into patients with the aim to restore, maintain, or improve tissue function. Cells can either be delivered as suspensions, or they are first grown *in vitro* into complete and functional tissue units in conjunction with biomaterial scaffolds, and then they are implanted once the engineered tissues have reached the desired properties. This latter technique is commonly referred to as tissue engineering. The potential impact of this field is significant—in the future, engineered tissues could reduce the need for organ replacement, and greatly accelerate the development of new drugs that may cure patients, eliminating the need for organ transplants altogether (Griffith and Naughton, 2002).

Blood transfusions, first successfully performed by James Blundell in 1818 to treat haemorrhages after childbirth, were the first type of cell therapy. Thereafter, in the late 1950s E. Donnall Thomas pioneered the development of bone marrow transplantation to treat leukaemia and other blood disorders. A decade later artificial skin for the treatment of burns was the first engineered tissue being developed (Hall et al., 1966, Spira et al., 1969). Unfortunately, the success of engineered dermal implants for treating skin injuries and burns has not been as easy to replicate for organs such as the liver and pancreas, partly because expanding hepatocytes or pancreatic islet cells in culture is much more difficult than expanding dermal fibroblasts or keratinocytes (Griffith and Naughton, 2002). Indeed, the source of replacement cells is of great importance for tissue engineering and ideally it should be readily available in sufficient quantity, not cause immunorejection and be safe for the recipient.

Recent advances in stem cell biology have opened new avenues for tissue engineering. Human embryonic stem (hES) cells are characterized by their capacity for self-renewal and their ability to differentiate into all cell types found in adult tissues. Furthermore, the surprising discovery by Takahashi and Yamanaka (Takahashi and Yamanaka, 2006) that somatic cells can be reprogrammed into induced pluripotent stem (iPS) cells, has allowed scientists to have access to unlimited, immunocompatible, and ethically acceptable cell sources. The potential of iPS cells in regenerative medicine is undeniable; these cells can be derived from essentially any individual and, after genetic modification, patient-derived iPS cells may be transplanted back into the patient for therapeutic purpose. However, the safety of any stem cell-based therapy is a paramount concern, since these cells are known to develop into tumours *in vivo*. Indeed, there are clear similarities between the excitement generated by cell therapy and tissue engineering today and that generated by the advent of gene therapy a few decades ago. Hopefully, the lessons learned from the difficulties and failures of gene therapy will be

applied to the nascent field of regenerative medicine and thus facilitate the development of safe and effective therapies (Porteus, 2011).

2.4.2 ENGINEERING VISION

Cell therapies for eye diseases are advancing rapidly and, in the anterior part of the eye, have already been tested in hundreds of patients. For example, limbal stem cell transplantation is successfully used in the clinical practice for the treatment of chemical burns to the cornea (Eveleth, 2013). In this medical procedure, a small sample of corneal limbus, containing the limbal epithelial stem cell niche, is surgically harvested from a donor eye, these cells are then expanded in culture and transplanted to the recipient cornea, alone or with a biomaterial scaffold, and eventually the transplanted epithelial cells will repopulate and heal the damaged cornea (Pellegrini et al., 1997, Rama et al., 2010).

As in the case of gene therapy, the eye is an ideal setting for the implementation of cell-based therapies, because a small number of transplanted cells may be sufficient to achieve a therapeutic effect, the transplantation of donor cells to precise locations is possible with help of direct microscopic visualization, the fate of transplanted cells can be followed in animals by non-invasive imaging techniques such as two-photon microscopy (Palczewska et al., 2014), allogenic cells are less likely to be rejected due to ocular immune privilege, and, due to the presence of several ocular barriers, the migration of transplanted cells outside the eye ball is unlikely.

There are a few cell therapies for retinal disease (dry AMD, Stargardt's disease, and retinitis pigmentosa) in clinical development, moreover the first ever hES cell-derived therapy has been assessed in humans for safety and tolerability (Schwartz et al., 2012). These therapies focus on the reconstruction of photoreceptors and retinal pigment epithelial cells using hES cells and retinal progenitor cells. In the case of AMD, the rationale behind the reconstruction of a functional RPE monolayer is twofold, firstly the transplanted RPE would replace dysfunctional RPE, and secondly the powerful trophic effect that the RPE exerts on photoreceptors may delay cell degeneration (Eveleth, 2013). In the above mentioned trials, the replacement cells are delivered subretinally as a suspension; this straightforward approach may however not guarantee the survival and function of the transplanted cells in the hostile environment in the diseased eye. Moreover, the cellular arrangement of the injected cells will be difficult, if not impossible, to control. For these reasons, the transplantation of a polarized RPE monolayer as an intact epithelial sheet supported by a carrier substrate may prove to be a better approach. The potential medical applications of tissue engineered RPE monolayers go beyond cell therapy; these constructs may also provide a unique platform from which to study disease, identify new

drugs, and screen for their toxicity and permeation across the outer blood-retinal barrier.

Another emerging AMD treatment currently in clinical trials combines the technologies of gene and cell therapy into one platform, encapsulated cell technology (ECT). ECT is a biotechnical implant system where genetically engineered cells that produce specific bioactive substances (i.e. proteins) are enclosed into polymer scaffolds to create “drug factories” for long-term drug delivery. Neurotech’s ECT intravitreal implant of encapsulated human retinal pigmented epithelial cells releasing ciliary neurotrophic factor (CNTF) is currently in a phase II clinical trial for macular degeneration.

2.5 PROTEIN THERAPEUTICS

2.5.1 CHALLENGES OF PROTEIN THERAPY

Compared to gene and cell therapy, protein therapeutics have enjoyed a much wider therapeutic success and are extensively used in the clinics to treat a broad range of illnesses, including cancer, autoimmune diseases, infectious diseases, and metabolic disorders. From a therapeutic perspective, proteins offer the distinct advantage of specific mechanisms of action and high potency (Pisal et al., 2010). Despite these attractive properties, proteins are notoriously challenging to develop and formulate due to chemical and physical instability, immunogenicity, and unfavourable pharmacokinetic properties. Protein stability can be compromised by several external factors such as pH, temperature, and surface interaction, as well as by contaminants and impurities from excipients (Frokjaer and Otzen, 2005), therefore stabilization of protein pharmaceuticals is of paramount importance to ensure their safety and efficacy. Indeed, protein instability, together with low permeability across biological membranes, are the two main reasons why proteins have to be administered parenterally rather than taken orally like most small molecule drugs. Moreover protein therapeutics must usually be stored at low temperatures or freeze-dried to achieve an acceptable shelf life (Wei, 1999). The pharmaceutical and pharmacokinetic properties of proteins can be optimized by different approaches; for example, by mutagenesis, chemical modification or by designing specific drug-delivery systems. However, most protein-based drugs today are still formulated as suspensions or aqueous solutions (Frokjaer and Otzen, 2005).

2.5.2 PROTEINS REVOLUTIONIZE THE TREATMENT OF WET AMD

Therapeutic proteins have found an extraordinary development in ophthalmology and intravitreal anti-angiogenic therapy has become the gold standard for the treatment of choroidal neovascularization in wet AMD. In 2006 the FDA approved ranibizumab (Lucentis), a 48 kDa fragment of a

recombinant monoclonal antibody that binds to and inhibits the vascular endothelial growth factor A (VEGF-A), for the treatment of wet AMD. Ranibizumab was the first therapy for neovascular AMD to result in vision loss prevention and moderate improvement in visual acuity (Rosenfeld et al., 2006, Brown et al., 2006).

Before the approval of ranibizumab, some ophthalmologists began administering bevacizumab (Avastin), a 149 kDa recombinant monoclonal antibody closely related to ranibizumab, off-label to treat patients with ocular neovascular diseases. Bevacizumab, which received its first approval in 2004 for the treatment of metastatic colorectal cancer, is manufactured by the same company as ranibizumab; however the low dosage needed in the eye (compared to the amounts required in colon and other cancers) make it much cheaper to use with similar efficacy (Martin et al., 2011).

Most recently, in 2011 aflibercept or VEGF Trap (Eylea), a recombinant soluble VEGF receptor protein (115 kDa) in which the binding domains of VEGF receptors 1 and 2 are combined with the Fc portion of immunoglobulin-G (Nguyen et al., 2006), has also been approved for CNV in wet AMD. Unlike pegaptanib, ranibizumab, and bevacizumab, which all act through inhibition of VEGF-A, aflibercept is designed to inhibit all members of the VEGF family (Chappelow and Kaiser, 2008), as well as placental growth factor 1 and 2 (Nguyen et al., 2006).

Ongoing clinical trials with therapeutic proteins for ocular diseases are focused not only on blocking neovascularization, but also on inhibiting complement pathway, on reducing inflammation and on conferring neuroprotection.

2.5.3 A CLOSER LOOK AT THE OCULAR PHARMACOKINETICS OF PROTEINS

Despite the widespread use and undisputed efficacy of intravitreally administered anti-VEGF antibodies, an obvious but not easily answered question remains: how are these large proteins and other biologics diffusing in the vitreous, penetrating the various retinal layers, and finally being eliminated from the eye? The diffusion of drugs in the vitreous is mainly dependent on their molecular weight and lipophilicity; due to their high molecular weight, biologics tend to have a slower diffusion and longer residence time in the vitreous compared to small molecule drugs. Moreover, due to the presence of hyaluronan, a negatively charged glycosaminoglycan, cationic macromolecules may aggregate and be immobilized in the vitreous, losing thereby their activity. This is the case with cationic non-viral gene delivery systems, which are completely inactivated when administered intravitreally (Pitkanen et al., 2004, Peeters et al., 2005).

The elimination of drugs from the vitreous follows first order kinetics and occurs by two major routes (figure 2): the anterior route (dashed arrows) via aqueous drainage through the anterior chamber, and the posterior route

(leftwards arrows), proposed to be the primary route for small and lipophilic molecules, as it requires adequate passive permeability or active transport across the retina and retinal pigment epithelium (Maurice and Mishima, 1984, Urtti, 2006). The posterior route causes a quicker elimination of the drug compared to the anterior route because the bottleneck between the lens and ciliary body is replaced by the large surface area in the posterior segment of the eye (Maurice and Mishima, 1984).

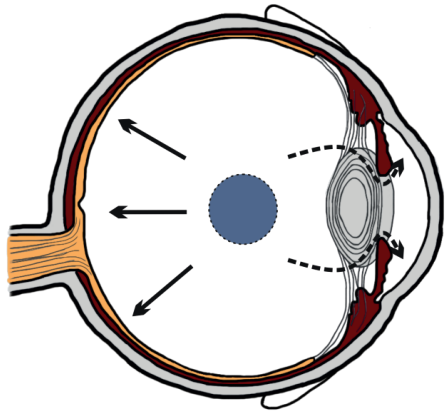

Figure 2 **Elimination of intravitreally administered drugs.** The posterior route (leftwards arrows) via the retina and RPE is favoured by small and lipophilic molecules, and promotes a quick elimination from the vitreous. This elimination pathway is precluded to hydrophilic drugs and macromolecules due to the biological barriers found in the ocular posterior segment. The anterior route (dashed arrows), through the posterior chamber, anterior chamber and via the trabecular meshwork to the canal of Schlemm, is accessible to all drugs, but it is slower due to the bottleneck between the lens and ciliary body.

Biologics are usually large hydrophilic molecules, therefore their elimination after intravitreal administration is expected to follow the less effective anterior route, and consequently their half-life in the vitreous tends to be prolonged compared to small molecule drugs. Nevertheless, studies with animal models have found that the full length antibody bevacizumab can permeate through all retinal layers and even reach the choroidal blood vessels (Shahar et al., 2006, Heiduschka et al., 2007), thereby challenging the classic assumption that large drug substances cannot be eliminated via retinal clearance. This observation may be explained by the presence of an active transport mechanism that promotes the elimination of antibodies from the eye (see below).

Unfortunately, comprehensive studies on human ocular pharmacokinetics of proteins and other biologics are lacking. Rabbits and monkeys are commonly used in ocular pharmacokinetic studies; however differences in ocular anatomy have led to criticism on the reliability of pharmacokinetic

data obtained from animal models. In humans, the vitreous volume is approximately 4 ml and, depending on age, its composition is 40% to 80% gel (Balazs, 1982, Sebag, 1987), whereas it is smaller in rabbits (1.5 ml) and monkeys (1.5-3.2 ml), but with higher vitreous gel content (100% in rabbits and 60% in rhesus monkeys). These interspecies differences have been used to explain the shorter vitreal half-lives of VEGF-inhibitors obtained from animal experiments compared to human data (table 4).

Table 4. *Intravitreal half-lives of anti-VEGF inhibitors in humans and rabbits.*

Drug	Mw (kDa)	$t_{1/2}$ in humans (days)	$t_{1/2}$ in rabbits (days)
Pegaptanib	50	8 (Basile et al., 2012)	3.5 (Martin et al., 2002)
Ranibizumab	48	7.2-9 (Krohne et al., 2012, Xu et al., 2013)	2.9 (Bakri et al., 2007a, Gaudreault et al., 2007)
Bevacizumab	149	6.7-10 (Krohne et al., 2008, Zhu et al., 2008)	4.3-6.0 (Bakri et al., 2007b, Nomoto et al., 2009)
Aflibercept	115	no published data	4.6 (Christoforidis et al., 2012)

Del Amo and colleagues (E. del Amo, K.S. Vellonen, H. Kidron, A. Urtti, In Silico Prediction of Intravitreal Primary Pharmacokinetic Parameters and Drug Concentrations: Tool for Ocular Drug Development. Pharm Res, *submitted*), however argue that the anatomical differences between the rabbit and human eye do not compromise ocular pharmacokinetic data and indeed, the reported differences in the half-lives are fairly small. The intravitreal half-life $t_{1/2}$ of a drug in the vitreous is described by following equation:

(1) $$t_{1/2} = (\ln 2 \times V_{ss})/Cl$$

where V_{ss} is the intravitreal volume of distribution and Cl is the intravitreal clearance. V_{ss} is expected to be approximately two times greater in the human eye compared to the rabbit eye. The Cl in the human eye is also expected to be increased 2-3 fold based on the larger RPE surface area, therefore the higher V_{ss} and Cl values may compensate for each other, leading to similar half-lives.

Table 4 also shows that, unexpectedly and despite the 3-fold difference in molecular weight, the intravitreal half-lives of ranibizumab (48 kDa) and bevacizumab (149 kDa) in humans are similar. Since the diffusion and elimination of a drug from the vitreous depend on its molecular weight, as well as its lipophilicity, one would expect that the larger full-sized IgG

bevacizumab would have a longer residence time in the vitreous compared to ranibizumab. This conflicting observation has been explained by the activity of the neonatal Fc receptor, which is known to bind IgG antibodies and transcytose them across tissue barriers (Rodewald and Kraehenbuhl, 1984, Simister and Rees, 1985). RPE cells express ocular neonatal Fc receptor (van Bilsen et al., 2011), therefore it is reasonable to assume that this active outwards transport mechanism from the retina to the blood circulation may contribute to a quicker elimination of bevacizumab, but not ranibizumab, from the vitreous. Furthermore the recycling function of neonatal Fc receptor on full-sized antibodies, may also explain the much longer plasma half-life of bevacizumab (20 days) (Lu et al., 2008) compared to ranibizumab (2 hours) (Xu et al., 2013). This very long systemic exposure to bevacizumab may lead to systemic inhibition of VEGF activity and undesirable side effects; indeed studies on patients receiving intravitreal injections of bevacizumab have shown suppression of systemic VEGF over a time period of four weeks (Carneiro et al., 2012, Matsuyama et al., 2010, Sato et al., 2012, Zehetner et al., 2013).

In conclusion, despite the hundreds of thousands intravitreal injections of therapeutic proteins performed every year, the knowledge on their ocular pharmacokinetics, the influence of ageing and pathological conditions, and the possible effects of repeated intravitreal injections, is still lacking. This knowledge is essential to implement the design of controlled delivery systems, as well as to develop new biologics with improved ocular pharmacokinetic properties.

3 AIMS OF THE STUDY

The thesis deals with the delivery of various biologics, including DNA, cells, and proteins to the retinal pigment epithelium, a monolayer of cells located underneath the retina in the back of the eye. The RPE is an interesting target from a pharmacological point of view, because RPE degeneration plays a central role in the pathogenesis of several degenerative retinal disorders that lead to irreversible vision loss. The specific aims were:

1. The development of a standardized transfection protocol optimized for the efficient and reproducible non-viral gene transfer to RPE cells *in vitro*.

2. The transfection of RPE cell models (ARPE-19, human primary RPE, and human embryonic stem cell-derived RPE) with non-viral gene delivery systems using the protocol developed in point 1. Transfection efficacy may be increased by the implementation of optimized genetic material and different carriers.

3. The clarification of the cellular uptake mechanism of Tat peptide, a cell penetrating peptide commonly used for the delivery of biologics. The structure-activity relationship of Tat peptide is investigated by modifications of its amino acid sequence.

4. The reconstruction of a functional RPE monolayer *in vitro*, by differentiation of human embryonic stem cells toward RPE on a transplantable, biopolymer coated polyimide membrane.

5. The evaluation of the cytoprotective properties of heat shock protein 70 kDa (Hsp70) against oxidative stress in the RPE, and the feasibility of Hsp70 protein therapy as a therapeutic strategy to target aggregate-associated neurodegeneration in AMD.

4 OPTIMIZED TRANSFECTION PROTOCOL FOR EFFICIENT *IN VITRO* NON-VIRAL POLYMERIC GENE DELIVERY TO HUMAN RETINAL PIGMENT EPITHELIAL CELLS (ARPE-19).

5 TAT(48-60) PEPTIDE AMINO ACID SEQUENCE IS NOT UNIQUE IN ITS CELL PENETRATING PROPERTIES AND CELL-SURFACE GLYCOSAMINOGLYCANS INHIBIT ITS CELLULAR UPTAKE.

Reprinted from Journal of Controlled Release, Vol 158, Astrid Subrizi, Eva Tuominen, Alex Bunker, Tomasz Róg, Maxim Antopolsky, Arto Urtti, Tat(48-60) peptide amino acid sequence is not unique in its cell penetrating properties and cell-surface glycosaminoglycans inhibit its cellular uptake, 277-285, Copyright 2012, with permission from Elsevier.

6 GENERATION OF HESC-DERIVED RETINAL PIGMENT EPITHELIUM ON BIOPOLYMER COATED POLYIMIDE MEMBRANES.

Reprinted from Biomaterials, Vol 33, Astrid Subrizi, Hanna Hiidenmaa, Tanja Ilmarinen, Soile Nymark, Peter Dubruel, Hannu Uusitalo, Marjo Yliperttula, Arto Urtti, Heli Skottman, Generation of hESC-derived retinal pigment epithelium on biopolymer coated polyimide membranes, 8047-8054, Copyright 2012, with permission from Elsevier.

7 OXIDATIVE STRESS PROTECTION BY EXOGENOUS DELIVERY OF RHHSP70 CHAPERONE TO THE RETINAL PIGMENT EPITHELIUM (RPE), A POSSIBLE THERAPEUTIC STRATEGY AGAINST RPE DEGENERATION.

8 SUMMARY OF THE MAIN RESULTS

The main results presented in the publications are summarized in table 5.

Table 5. *Summary and implications of the main results.*

RPE transfection protocol (publication I)	
Optimal parameters	20´000 cells/well (96 wp), PEI/DNA ratio 2/1 (= n/p 10), Mes-Hepes buffer, 1-2 h incubation with cells.
Expected outcome	10-20 ng/ml RL protein (DNA dose 600 ng), 60-80% cell viability.
This protocol provides a relatively simple and reproducible procedure for the pre-selection of potential candidate reagents as non-viral gene delivery systems to the RPE.	

Cell penetrating properties of Tat peptide (publication II)	
Interaction with model membrane	Could not predict the cellular behaviour of Tat peptide.
Amino acid sequence	May be modified without loss of activity, as long as the number of positive charges does not change.
Cell uptake	Occured by endocytosis, possibly also phagocytosis. No direct translocation across the plasma membrane was observed.
Effect of glycosaminoglycans	Inhibited Tat upake.
The usefulness of Tat peptide as a tool for the delivery of biologics is questionable due to the lack of cellular specificity and lysosomal entrapment.	

RPE tissue engineering (publication III)	
Polyimide membrane suitability	Required coating with bioadhesive molecules to support growth and maturation of hESC-RPE.
Bioadhesive molecules	Laminin and collagen type I and IV provided suitable coating.
Characteristics of tissue engineered RPE	Cobblestone morphology, highly pigmented, polarity (apical localization of Na/K-ATPase and MERTK), expression of RPE-specific marker proteins (MITF, PMEL, TYR, RPE65, BEST, PEDF, CRALBP), formation of tight junctions (ZO-1) and barrier function which was moderately leakier than the normal RPE tissue, phagocytic activity.
hESC-RPE grown on polyimide membranes may be useful for in vitro drug screening, cell replacement therapy and disease model development, provided that the time required to generate these tissues is shortened.	

Hsp70 therapy against oxidative stress in the RPE (publication IV)	
Effect of Hsp70 treatment	Hsp70 protected ARPE-19 cells from oxidative stress by reducing inflammation, increasing cell viability, and decreasing cytolysis.
In vitro uptake and localization of Hsp70	Exogenously delivered Hsp70 was internalized by dividing and differentiated ARPE-19 cells and was localized in late endosomes and lysosomes.
Intraocular distribution *ex vivo*	Intravitreally administered Hsp70 diffused to the retina and the RPE.
Hsp70 therapy may provide a possible therapeutic strategy against degeneration of RPE cells in AMD.	

9 UNPUBLISHED RESULTS

In addition to the published work, following unpublished transfection results are also included in the thesis. Transfections were carried out following the published protocol (publication I). ARPE-19 human retinal pigment epithelial cell line, human primary RPE cells, and hESC-RPE human embryonic stem cell-derived RPE, were used as RPE cell models. Optimization of the genetic material was achieved using three different strategies: 1) introduction of genetic elements favouring the episomal replication of the plasmid (EBNA-1 plasmid) (Hung et al., 2001); 2) use of the alternative non-viral promoter human elongation factor-1 alpha (EF1a); and 3) elimination of bacterial plasmid DNA sequences (minicircles) (Kay, 2011). Different carriers, including cationic DOTAP/DOPE/PS fusogenic liposomes (Mannermaa et al., 2005) and amphiphilic block copolymer core-shell micelles (Alhoranta et al., 2011) were tested for transfection efficacy and compared to branched polyethylenimine (PEI, 25 kDa) (Boussif et al., 1995). The size of the carrier/DNA complexes was 100-250 nm. The carrier to DNA ratios were chosen after observation of the complexation on electrophoresis gels and after following the release of DNA from the complexes upon exposure to dextran sulphate (Xu and Szoka, 1996). A selection of the transfection results is provided.

9.1 EFFECT OF EBNA-1 ON THE TRANSFECTION OF DIFFERENTIATED ARPE-19 CELLS GROWN ON LAMININ-COATED TRANSWELL FILTERS

The introduction of EBNA-1 genetic elements (figure 3) prolonged the secretion of the transgene (dashed lines) even in non-dividing cells, compared to non-replicating plasmid (solid lines).

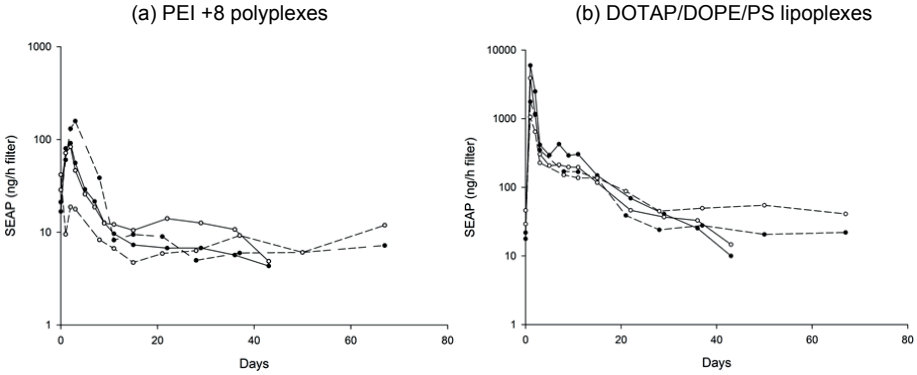

Figure 3 **Effect of EBNA-1 on transfection of differentiated ARPE-19 cells.** Transgene secretion curves of cells transfected with EBNA-1 plasmids are presented as dashed lines, in comparison, transgene secretion curves of cells transfected with regular non-episomal plasmids are presented as solid lines. The full symbols represent apical secretion and the empty symbols represent basolateral secretion. (a) Transfection with PEI polyplexes, and (b) transfection with dotap/dope/ps lipoplexes.

9.2 MINICIRCLE EFFICACY AND INFLUENCE OF PROMOTER COMPARED TO EBNA-1 PLASMID ON THE TRANSFECTION OF HESC-RPE CELLS

The transfection with minicircles increased transgene production compared to plasmid DNA containing EBNA-1 genetic elements. For PEI polyplexes (figure 4 a), the secretion of the transgene decreased significantly two weeks after transfection, while lipoplexes (figure 4b) still maintained a detectable protein secretion level 20 days after transfection. In the case of polyplexes, the use of non-viral promoter EF1a did not prevent promoter shutdown; in the case of lipoplexes, instead, the use of EF1a promoter drove stable transgene secretion for up to 20 days.

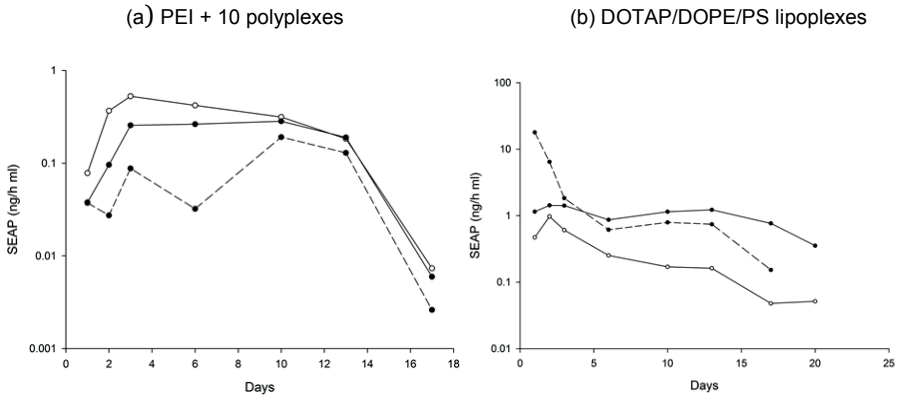

Figure 4 **Effect of minicircle vs regular plasmid (EBNA-1) and influence of the promoter on transgene secretion.** Transgene secretion curves of cells transfected with EBNA-1 plasmids are presented as dashed lines, transgene secretion curves of cells transfected with minicircles are presented as solid lines. The empty symbols represent minicircles with viral CMV promoter, the full symbols represent minicircles with human EF1a promoter. (a) Transfection with PEI polyplexes, and (b) transfection with dotap/dope/ps lipoplexes.

9.3 AMPHIPHILIC BLOCK COPOLYMER CORE-SHELL MICELLES PROMOTE HIGHER TRANSFECTION EFFICIENCY AND PROTEIN SECRETION FOR UP TO 18 DAYS IN HUMAN PRIMARY RPE

Amphiphilic block copolymer core-shell micelles (dashed lines) showed increased transfection efficacy in human primary RPE compared to PEI polyplexes (solid lines, full symbol) and DOTAP/DOPE/PS lipoplexes (solid lines, empty symbol): cells secreted higher amounts of transgene for a longer period of time (figure 5).

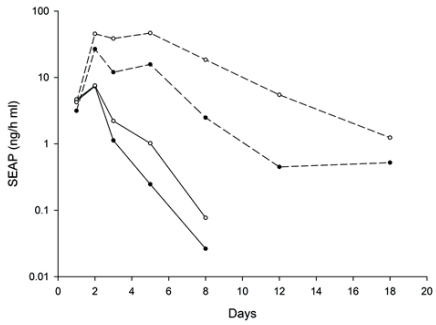

Figure 5 **Effect of carrier on the transfection of human primary retinal pigment epithelial cells.** Transgene secretion curves of cells transfected with minicircles carrying CMV promoter. Cells transfected with micellar nanoparticulates, linear (full symbol) and star (empty symbol) poly(n-butyl acrylate) – 2-(dimethylamino)-ethyl methacrylate, PDMAEMA-PBuA-PDMAEMA and $(PBuA\text{-}PDMAEMA)_6$, respectively, are presented as dashed lines. In comparison, PEI polyplexes (solid line, full symbol) and DOTAP/DOPE/PS lipoplexes (solid line, empty symbol).

10 DISCUSSION

Biologics are a new category of drugs, which have emerged rapidly in the drug discovery pipeline in the last two decades. The first insulin-related biologics that came to the market already 30 years ago, have paved the way for the development of a wide variety of biotechnologically produced drugs, including monoclonal antibodies, growth factors, genetic material and cell therapeutics. Since the approval in 2002 of adalimumab (Humira), a TNFα inhibitor for the treatment of rheumatoid arthritis, human or humanized monoclonal antibodies are the most prevalent and fast-growing category of biologics (Nelson et al., 2010). Indeed in the field of ophthalmology, the humanized monoclonal antibody/antibody fragment bevacizumab and ranibizumab are the therapy of choice for the treatment of age-related macular degeneration, a disorder of the posterior eye and leading cause of blindness in the elderly in the western world. Moreover biologics have also been used off-label to treat another vision threatening disorder, uveitis or ocular inflammation disease (Lim et al., 2006, Imrie and Dick, 2007).

This thesis focuses on the delivery of different biologics to the retinal pigment epithelium, the monolayer of pigmented cells joined apically with tight junctions that forms the outer blood-retinal barrier in the eye. The RPE is an interesting target for biologics because its function is essential for retinal homeostasis and RPE atrophy leads to irreversible vision loss. Given its strategic position between the retina and the choroidal blood vessels, multiple therapeutic interventions targeting the RPE can be envisioned; 1) the transfection of transgenes coding for neurotrophic factors may help to maintain the viability of photoreceptors in the retina, while anti-angiogenic factors may prevent the uncontrolled growth of blood vessels from the choroid, 2) the delivery of therapeutic proteins may prevent RPE degeneration, and 3) a tissue engineered RPE monolayer may provide a replacement, if the tissue *in situ* is beyond repair. In the following paragraphs a general discussion touching upon these three main therapeutic approaches is presented.

Non-viral ocular gene transfer to the RPE after intravitreal injection is challenging; polyplexes and lipoplexes have to travel a relatively long distance from the administration site and numerous ocular barriers may prevent their arrival to the target cells (Peeters et al., 2007). The first barrier encountered by the complexes is the vitreous itself; this complex and negatively charged three-dimensional network of collagen fibrils, glycosaminoglycans, and noncollagenous structural proteins, is known to immobilize cationic nanoparticulate systems and completely block gene expression of polyplexes and lipoplexes (Pitkanen et al., 2003, Peeters et al., 2005). The mobility of cationic complexes in the vitreous can be improved through PEGylation (Peeters et al., 2005, Bochot et al., 2002); indeed

pegaptanib (Macugen), the 28-base RNA aptamer and only gene medicine approved so far for ocular treatment, is covalently linked to two branched 20 kDa PEG moieties that increase its intravitreal half-life and the sugar backbone has been modified to prevent nuclease degradation (Ruckman et al., 1998). Another barrier encountered by non-viral gene delivery systems in their journey from the vitreous to the RPE is the neural retina; the retina limits the delivery of polymeric and liposomal DNA complexes to the RPE (Pitkanen et al., 2004), possibly by steric hindrance. The final extracellular barrier consists in the glycosaminoglycans found in the interphotoreceptor matrix and on the surface of RPE cells. RPE cells constantly synthesize and secrete glycosaminoglycans such as chondroitin sulphate, dermatan sulphate, and hyaluronic acid (Edwards, 1982); and these polyanions have been found to pose an important barrier to non-viral gene transfer (Ruponen et al., 2004).

Cell penetrating peptides (CPPs) are often employed to deliver biologics, genes and proteins, to the desired targets *in vitro* and *in vivo* (Gait, 2003, Lindsay, 2002, Zhao and Weissleder, 2004, Schwarze et al., 1999). Could coupling of biologics to CPPs facilitate their delivery to the RPE after intravitreal injection? Or in other words, can the intraocular barriers be overcome with the help of CPPs? The most intensely studied yet least understood peptide is Tat peptide. Our study aimed at elucidating Tat peptide's mechanism of cell penetration, and at distinguishing the amino acids in its sequence that are essential for its function from those that can be changed without affecting cell permeation efficacy. After synthesizing sixteen Tat peptide analogues bearing the same charge as the original Tat sequence, and testing them for cellular uptake in three cell lines, we came to the conclusion that it is not the sequence of Tat *per se* that dictates cell uptake, but the cationic charge of the peptide. Upon confocal microscope inspection, all the peptides were trapped inside lysosomes and direct penetration across the cell membrane was not observed for any peptide. Furthermore, our study showed that, similarly to non-viral gene delivery systems, glycosaminoglycans inhibit the cellular uptake of Tat peptide. Unfortunately, these results do not vouch for the usefulness of Tat peptide in helping to overcome the intraocular barriers. On the contrary, its cationic charge may hamper its diffusion in the vitreous, as well as make the targeting of the biologic to the RPE more difficult, since any anionic structure on the surface of cells will attract the CPP and its cargo. Furthermore, biologics requiring cytoplasmic or nuclear delivery will be instead targeted to the endosomes and eventually be degraded inside lysosomes.

In order to avoid the ocular barriers discussed in the previous paragraphs, we chose to follow an *ex vivo* approach, where the transfection of RPE cells occurs outside the eye in a culture. This technique is also beneficial because it does not require targeting to a particular tissue, and the risk of adverse reactions due to an unintended dissemination of the vector is avoided. The first aim was to develop a standardized protocol optimized for the

transfection of retinal pigment epithelial cells (ARPE-19) *in vitro*. We employed a stepwise approach by addressing the following parameters and studying their effects on the final transfection outcome: cell density at seeding, PEI/DNA charge ratio, composition of the preparation buffer and polyplex assembly conditions, and incubation time of the complexes with cells (see table 6).

Table 6. *Parameters tested for the optimization of the transfection protocol.*

Tested parameter	Optimal parameter
Cell density at seeding *4'000, 8'000, 20'000 cells/well in a 96 wp*	20'000 cells/well gave the highest protein production and lowest cytotoxicity
PEI/DNA charge ratio[1] *1/1, 2/1, 4/1*	Charge ratio 2/1 was less toxic and more efficient
Polyplex assembly conditions *Diluted, concentrated (see text)*	No substantial differences. Diluted conditions allowed for better mixing.
Polyplex preparation buffer *mqH_2O, Mes-Hepes buffered saline*	Polyplexes prepared in Mes-Hepes buffered saline were more efficient.
Incubation time of the complexes with cells *30 min, 1 h, 2 h, 5 h, 12 h, 24 h*	1-2 h incubation time gave the best results

[1] Charge ratio 2/1 corresponds approximately to n/p ratio 10.

The cell density at seeding remarkably affected the transfection outcome, and 20'000 cells per well, which yielded a sub-confluent (80%) culture after overnight incubation, gave the highest transfection efficacy and lowest cytotoxicity. The charge ratio of the polyplex influences the DNA condensation and the complex stability; in our setup the best charge ratio was 2/1. With PEI based nanoparticulates a significant proportion of the polymer utilized in the initial formation of the polyplex remains in the free form (Hanzlikova et al., 2011), and this may be partly responsible for the cytotoxicity. The composition of the nanoparticulate preparation buffer (pH and ionic strength) strongly affected gene expression; a higher level of transfection (500-fold) was achieved when polyplexes were prepared in isotonic Mes-Hepes buffered saline solution compared to distilled water. The preparation of the nanoparticulates in a more concentrated (i.e. mixing of 250 µl cationic polymer with 250 µl plasmid DNA, with final dilution of the nanoparticulates at the desired volume after 2 h incubation at room temperature) versus diluted (i.e. mixing of 500 µl cationic polymer with 500 µl plasmid DNA) conditions showed little effect on the transfection efficiency; however, especially in the case of small preparation volumes, we preferred to add the cationic polymer to plasmid DNA in diluted conditions because mixing was more effective. The incubation time of the nanoparticulates with ARPE-19 cells was of crucial importance to obtain high gene expression levels and minimal cytotoxicity; we found that an incubation time of 1 to 2 hours was optimal for our application. If shorter time was

applied, transfection efficiency was poor, while longer incubation periods caused cell toxicity.

The optimized transfection protocol was utilized to test different carriers, including cationic DOTAP/DOPE/PS fusogenic liposomes (Mannermaa et al., 2005), and amphiphilic block copolymer core-shell micelles (Alhoranta et al., 2011), with RPE cells. The human retinal pigment epithelial cell line ARPE-19, primary human RPE and human embryonic stem cell-derived RPE (hESC-RPE) were used. Branched PEI 25 kDa was used as positive control. One of the shortcomings of non-viral gene transfer is that it tends to be short lived and the expression of the transgene is lost after a couple of days; however the expression of a curative transgene *in situ* should last months, if not years, in order to exert a lasting therapeutic effect. We have employed three different strategies aimed at the optimization of the genetic material to prolong gene expression; 1) the loss of plasmid was minimized by the addition to the plasmid sequence of genetic elements from Epstein-Barr virus (EBV), EBNA-1 and OriP that promote episomal replication (Hung et al., 2001), 2) promoter shutdown was prevented by the use of the human promoter elongation factor-1 alpha (EF1a), and 3) the loss of transgene-specific mRNA was minimized by the use of minicircle DNA that is devoid of bacterial plasmid DNA sequences and has lower CpG content (Kay, 2011).

With these improved non-viral gene delivery systems we could transfect all RPE cell models, including the notoriously hard to transfect stem cells. The use of EBNA-1 plasmids was advantageous compared to non-replicating plasmids, as it considerably increased the length of transgene production for up to 2 months in non-dividing ARPE-19 cells. Furthermore minicircles were generally equally or better performing compared to EBNA-1 full plasmids, and the use of non-viral promoter EF1a further enhanced protein secretion time depending on the carrier used. The activity of different carriers was cell-specific, and in the case of human primary RPE, amphiphilic block copolymer core-shell micelles were far superior compared to PEI polyplexes and DOTAP/DOPE/PS lipoplexes.

These studies show that, despite being still inferior to viruses, non-viral gene delivery systems can be improved and optimized for the required needs; however their activity is cell-specific and a generalization for other tissues may not be possible. Unfortunately, there does not seem to be such thing as a "one fits all" delivery system. In conclusion, *ex vivo* gene therapy to the RPE may be a successful approach, especially if combined with RPE tissue engineering; the genetically modified and reconstructed RPE monolayer may replace the tissue *in vivo*, support the neural retina with neurotrophic factors, and also secrete antiangiogenic proteins to prevent neovascularization.

The reconstruction of the RPE is a valid therapeutic option in the case of advanced AMD patients, where scarring and tissue atrophy have already occurred. It is worthwhile remembering that 95% of all AMD patients have currently no cure available to treat the disease and prevent vision loss.

Several attempts have been made to replace the diseased RPE with functional RPE (Schraermeyer et al., 2001, Lu et al., 2009, Idelson et al., 2009, Lund et al., 2006), however in these studies the replacement cells were injected subretinally as a suspension and without any support, which would promote the correct cellular arrangement and protect the transplanted cells from the hostile environment found in AMD eyes

In our study we used human embryonic stem cell-derived RPE (hESC-RPE) as cell source and the synthetic non-biodegradable polymer polyimide as RPE carrier substrate. The safety and tolerability of hESC-RPE cell therapy in humans has been recently assessed in a clinical trial (Schwartz et al., 2012), while polyimide is already clinically approved for ocular use and its biocompatibility has been demonstrated in several ophthalmic applications (Kane et al., 2008, Besch et al., 2008, Julien et al., 2011). The cells adopted RPE like phenotype with intense pigmentation, expressed melanogenesis-related genes microphthalmia-associated transcription factor (MITF), pre-melanosome protein 17 (PMEL), and tyrosinase (TYR), and RPE-specific genes retinal pigment epithelium-specific 65 kDa protein (RPE65), bestrophin-1 (BEST), pigment epithelium-derived factor (PEDF), and cellular retinaldehyde-binding protein (CRALBP). Furthermore, the monolayers exhibited polarity with apical expression of Na/K-ATPase and C-mer proto-oncogene tyrosine kinase (MERTK), expressed the tight junction protein 1 (ZO-1), and phagocytosed shed outer segments. The RPE monolayers exhibited a barrier function which was moderately leakier than the normal RPE tissue. One drawback of hESC-RPE monolayers grown on polyimide membrane is the very long differentiation and maturation time (several months) required to achieve a functional RPE; shortening of the protocols used to engineer the RPE monolayers would make them more appealing from a therapeutic point of view, as well as for their use as RPE cell models for *in vitro* drug screening.

The transplantation of a tissue engineered RPE monolayer is expected to be beneficial when the photoreceptors are still intact; in this case the transplanted RPE may prevent photoreceptor cell loss and preserve the viability and function of the neural retina. If, however, photoreceptor cell death has already occurred, replacement of the RPE alone may not be sufficient. In this case, in addition to the RPE, also the photoreceptor cell layer must be reconstructed. In both cases such treatments are likely to be very costly and, considering the large number of potential patients, their future implementation in the current standards of care for AMD may be economically unsustainable.

The treatment of neovascularization in wet AMD has improved enormously since the introduction of anti-neovascular agents; however the much more common dry AMD remains still untreatable. Recently, the chronic exposure to oxidative stress and a decline in lysosomal activity of RPE cells have been recognized as a possible cause for RPE atrophy in dry AMD (Winkler et al., 1999, Kaarniranta et al., 2013, Jarrett and Boulton,

2012). Dry AMD is characterized by protein misfolding, accumulation of protein aggregates inside and underneath the RPE, and abnormal protein degradation that eventually lead to the degeneration of the RPE. Heat shock proteins (Hsps) are responsible for the correct folding of proteins and members of the Hsp70 family have the remarkable ability to dissolve protein aggregates; therefore Hsp70 protein therapy may protect RPE cells from oxidative stress and degeneration. Our study showed that Hsp70 therapy protects RPE cells from oxidative harm, and that the protein can be delivered to the RPE *in vitro* and also after intravitreal administration in an isolated porcine eye. Clearly this type of protein therapy needs to be started early in AMD patients, when the RPE cell layer is not yet compromised. Nevertheless, this work may hopefully provide a novel therapeutic option for the treatment of RPE degeneration in AMD.

The approaches mentioned so far for the treatment of AMD are suffering from one major flaw: they are invasive and in some cases they require repeated administrations. The subretinal transplantation of a tissue engineered and genetically modified RPE monolayer is a difficult technique that requires surgery and dedicated medical personnel with special skills. Intravitreal injections are easier to perform and currently carried out in an ambulatory setting, however the discomfort for the patient is not to be undervalued, especially considering that these injections are given monthly or even more often. Therefore, delivery systems that can efficiently target the diseased ocular tissues, generate therapeutic drug levels, and maintain prolonged and effective concentrations are highly desirable. The major hurdle in designing such delivery systems is to ensure that the structure and activity of biologics are retained during the preparation, sterilization and release processes (El Sanharawi et al., 2010).

Ocular drug delivery systems fall into five categories: implants, microspheres, nanoparticulates, cell encapsulation (ECT) (discussed previously), and hydrogels (del Amo and Urtti, 2008). Implants, which can be biodegradable or non-biodegradable, are positioned intravitreally with a minor surgery and are designed to release the encapsulated drugs for a prolonged period of time. Since 1996, four implants have been approved for the delivery of small molecule drugs to the back of the eye, Vitrasert (withdrawn from the market in 2013), Retisert, Ozurdex, and Iluvien. Recently, pSivida, the company involved in the development of three of the commercially available implants, has designed a delivery system (Tethadur) for the sustained delivery of bevacizumab. The implant, made of nanoporous silicon, controls the release of the antibody through its pore size.

Sustained drug release can also be obtained when the drug is encapsulated into microspheres (1-1000 μm). Biodegradable microspheres composed of biocompatible and FDA approved polymers poly (lactic acid) (PLA), poly (glycolic acid) (PGA) and their copolymers poly (lactic-co-glycolic acid) (PLGA) are the most employed. These polymers have different degradation rates (PLGA>PLA and PGA), therefore, by varying the ratio and

the molecular weight of the polymers used in the formulation, the degradation rate of the microspheres can be controlled. After intravitreal injection, these microspheres tend to aggregate and sink to the lower part of the vitreal cavity (Giordano et al., 1995, Herrero-Vanrell and Refojo, 2001, Cardillo et al., 2006, Barcia et al., 2009), therefore the risk of vitreal clouding is reduced. PLGA microspheres have been used in animal models to deliver biologics to the back of the eye. Intravitreally administered PLGA microspheres delivering glial cell line-derived neurotrophic factor (GDNF) protected retinal ganglion cells in a rat model of glaucoma (Jiang et al., 2007, Checa-Casalengua et al., 2011) and promoted photoreceptor survival in a retinal degeneration mouse (Andrieu-Soler et al., 2005). PLGA microspheres were also used to deliver an anti-VEGF RNA aptamer through a rabbit sclera *ex vivo*, the transcleral diffusion of the aptamer was followed for 6 days (Carrasquillo et al., 2003). Furthermore PLGA microspheres loaded with PEI complexed to antisense TGF-B2 oligonucleotides and administered by subconjunctival injection in rabbits, increased the intracellular penetration of the oligonucleotides in conjunctival cells and were able to prevent post-surgical fibrosis after glaucoma filtering surgery (Gomes dos Santos et al., 2006). One potential drawback of PLGA polymers is caused by their degradation into lactic and glycolic acid; these acidic molecules lead to a pH drop in the core of the microspheres that can compromise the stability of the encapsulated biologics (van de Weert et al., 2000, Zhu et al., 2000).

Nanoparticulates (1-1000 nm), such as polymeric nanoparticles, micelles, and liposomes, are self-assembling systems that have been extensively studied for the delivery of genetic material *in vitro* and *in vivo*. As discussed earlier, cationic nanoparticles may aggregate and be stuck in the negatively charged vitreous; however negatively charged PLA nanoparticles loaded with fluorophores were shown to diffuse rapidly in the vitreous, penetrate the retina and localize in the RPE for 4 months after a single intravitreal injection (Bourges et al., 2003). Furthermore PEDF peptides protected the rat retina from ischemic injury when delivered in PLGA nanospheres (Li et al., 2006). Sakai and colleagues (Sakai et al., 2007) evaluated the neuroprotective effect of bFGF-containing nanoparticles in RCS rats; the nanoparticles were present in several retinal layers and the RPE until 8 weeks after intravitreal administration and prevented photoreceptor degeneration. Liposomes have been shown to improve the intraocular pharmacokinetic properties of biologics. For example, the mean concentration of bevacizumab after intravitreal injection was twice higher at day 28 and 5 times higher at day 42, compared to free bevacizumab, if the antibody was encapsulated inside liposomes (Abrishami et al., 2009). The intravitreal concentration of a model 16-mer oligonucleotide was 9.3-fold higher when it was delivered inside liposomes compared to free oligonucleotide (Bochot et al., 2002), and also, 24 h after administration, the intravitreal amount of a vasoactive intestinal peptide encapsulated inside liposomes was 15 times greater than after injection of the peptide in saline

(Lajavardi et al., 2007). Another advantage of liposomes is that the encapsulated drugs appear to be less toxic, because only a limited amount of drug comes in direct contact with the ocular tissues (Short, 2008).

Hydrogels are made from polymers that upon a stimulus, such as temperature, pH, or ion concentration, undergo a phase change. These materials are liquid and can be delivered with a syringe, but once inside the eye they form a semi-solid matrix that retains the drug and releases it over time. Gel forming solutions are already in clinical use for the topical delivery of small molecule drugs (Timoptic-XE – timolol maleate, Pilogel – pilocarpine, and AzaSite – azithromycin) (del Amo and Urtti, 2008), however intravitreal gelifying systems for the controlled delivery of biologics still need further investigation.

Overall, the use of biological therapies to treat human disease is growing rapidly and millions of patients suffering from eye disorders, cancer, infectious diseases, inflammatory diseases, autoimmune disorders, cardiovascular diseases, blood disorders, and diabetes are successfully treated with biologics. Paradoxically, the success of biological therapies is starting to be a growing problem for public healthcare services worldwide, indeed, while these new therapies offer hope for the treatment of previously incurable diseases, they also tend to be much more expensive than conventional drugs, and managing their use is a challenge for payers (Kelly and Mir, 2009). One extreme case is Glybera, an AAV-based gene therapy for the rare genetic disease lipoprotein lipase deficiency that costs over € 1 million per patient. Healthcare costs are on an unsustainable trajectory in most developed countries, and this trend is expected to continue in the coming years with an aging population, and the growing incidence of lifestyle diseases. Therefore, the management of healthcare costs without having to compromise the access to quality medical care remains one of the most daunting challenges that governments are faced with. Many factors contribute to the high cost of biologics, including the complex manufacturing processes, and the fact that no other treatment may be available for a certain medical condition. Furthermore, most biologics cannot be taken by the patient directly, but instead, they have to be administered parenterally in a hospital thereby also contributing to the overall expenditure.

Given that the first biologics were approved in the 1980s, however, the exclusive rights, including patents and other data protection, for several biological medicinal products have reached their expiration. Consistent with this expiry, generic biological medicinal products, or biosimilars as they are commonly called, are being developed and several are already available on European markets, with the first approved and marketed in 2006. As their name implies, biosimilars, unlike generic drugs where the active ingredients are identical, are similar but not identical copies of the original biologic. Indeed, the inherent complexity of biologics precludes identical copies; similar to snowflakes, biosimilars from different manufacturers will differ from the original biologic and from each other. Nevertheless, according to

the EMA a biosimilar and the original biologic are expected to have the same safety and efficacy profile. Biosimilars may offer a less-costly alternative to existing biologics; as a result, their increased availability may improve access to biological medicines for more patients and contribute to the financial sustainability of healthcare systems. Thus, the development of biosimilars offers potential economic benefit to healthcare systems while addressing the issue of new treatment options brought about by advances in medical science.

11 CONCLUSIONS

In this thesis, the delivery of various biologics, including DNA, cells, peptides and proteins to the retinal pigment epithelium was explored. In response to the aims at page 28, the conclusions of this study are as following:

1. A standardized transfection protocol was developed and systematically optimized to obtain reproducible, high-level gene transfer to retinal pigment epithelial cells (ARPE-19) *in vitro*, using branched polyethylenimine (PEI) as a gene carrier. The development of the protocol involved the optimization of several parameters such as cell density at seeding, PEI/DNA charge ratio, composition of the preparation buffer and nanoparticulate assembly conditions as well as incubation time of the nanoparticulates with ARPE-19 cells. The selection of the most effective conditions for gene transfer led to the finalization of the present protocol that provides a relatively simple and reproducible procedure for the pre-selection of potential candidate reagents as non-viral gene delivery systems to the RPE.

2. The transfection protocol developed in point 1 was used to transfect ARPE-19 cells, human primary RPE, and hESC-RPE cells. Transfection efficacy and duration increased when optimized genetic materials including episomal EBNA-1 plasmid, human promoter, and minicircles were used. Several carriers including lipoplexes and amphiphilic block copolymer core-shell micelles showed promising results compared to branched PEI.

3. In our work we explored the functionality of Tat peptide, one of the most widely studied CPPs. Our results indicated that it is not the sequence of Tat *per se* that dictates cell uptake, but the cationic charge of the peptide. These cationic peptides seem to act via ionic interactions that should be operative regardless of the sequence and trigger peptide endocytosis Moreover no direct penetration was observed; instead the peptides were endocytosed and, as it is often the case in non-viral gene delivery, ended their journey inside lysosomes. Cell-surface glycosaminoglycans inhibited Tat peptide internalization. Based on our results we think that Tat peptide may not hold promise for the delivery of biologics to the RPE due to the unspecific mode of action and inability to escape from the endosomal compartments.

4. Ocular stem cell therapy holds promise for the reconstruction of the degenerated RPE monolayer in AMD patients; in addition, engineered human RPE constructs may also provide a unique platform for drug

discovery and toxicology. We have grown a functional RPE tissue *in vitro* by using human embryonic stem cells as cell source and the synthetic polymer polyimide as supporting scaffold for the growth and maturation of the cells. Human embryonic stem cell-derived RPE successfully matured on polyimide biomembranes and acquired RPE-like properties, including characteristic RPE phenotype, expression of RPE markers, barrier and phagocytic function.

5. RPE degeneration in dry AMD is caused by the aggregation of proteins inside RPE cells, and is currently untreatable. We investigated the cytoprotective properties of heat shock protein 70 kDa (Hsp70) against oxidative damage and the feasibility of rhHsp70 protein therapy as a potential therapeutic approach for dry AMD. Heat shock protein 70 kDa therapy protected the RPE from oxidative stress. The exogenously delivered protein was internalized into RPE cells, localized inside late endosomes and lysosomes, and was found to diffuse to the outer ocular layers, including the RPE, after intravitreal administration in *ex vivo* eyes. These results may hopefully provide a valid therapeutic strategy for the treatment of RPE degeneration, which is at the basis for the development of AMD.

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